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***142640** Links

HISTIDINE-RICH GLYCOPROTEIN; HRG

Alternative titles; symbols

HRGP
THROMBOPHILIA DUE TO ELEVATED HRG, INCLUDED

Gene map locus 3q27

TEXT

Histidine-rich glycoprotein is a nonenzymatic protein present in plasma and platelets. Its protein structure was characterized by Koide et al. (1986). Leung (1993) characterized HRG as an abundant plasma protein in search of a function. Although the physiologic role of HRG has not been established, several biologic properties of the protein have been identified. Properties that are relevant to the hemostatic mechanism are its binding to plasminogen and to heparin. By binding to plasminogen, HRG reduces the amount of plasminogen in the circulating blood that is available for activation into plasmin and thus acts as an inhibitor of fibrinolysis. By binding to heparin, HRG reduces the amount of heparin and through this mechanism reduces the inhibition of coagulation by the heparin-antithrombin III complex. Since both effects, the inhibition of fibrinolysis and the reduction of inhibition of coagulation, work in the same direction, an excess of HRG might be expected to have a prothrombotic effect. Engesser et al. (1987) described a family with thrombophilia and elevated levels of HRG in 5 persons in 3 sibships of 2 generations. By history a third generation was affected, and the apparent involvement of a deceased member represented male-to-male transmission. Falkon et al. (1992) described a similar family in which high levels of the HRG protein was demonstrated. The family was brought to attention by a 41-year-old woman who had had recurrent thromboembolic disease starting at the age of 22 years. She was also shown to have elevation of plasminogen activator inhibitor-1 (PAI1; 173360), which was not detected in any other member of the family. Falkon et al. (1992) suggested that elevated HRG may not be a strong risk factor for venous thrombosis when it is not combined with another defect. Angles-Cano et al. (1993) found high levels of HRG and PAI1 in 8 and 10 members, respectively, of a family from which 4 of 7 members with both abnormalities had venous thromboembolism. On the basis of their studies, they concluded that the excess HRG was probably not related to the thromboembolic events.

Hoffmann et al. (1993) found persistently elevated levels of plasma HRG in a 64-year-old female with a history of recurrent arterial thromboembolic events. Increased HRG was found in 8 of 17 relatives studied, but none of them had experienced thromboembolism. Increased HRG was inherited as an autosomal dominant. The plasma HRG of the proposita and 9 of her family members displayed abnormal binding to heparin, as assessed in a crossed affinity immunoelectrophoresis system; the usual increase in mobility after binding to heparin was absent. The binding of the variant HRG to plasminogen was normal. This was the first example of an abnormal HRG variant. The designation HRG Eindhoven was proposed.

Using a cDNA for HRG isolated by Koide et al. (1986) to screen a panel of rodent-human somatic cell hybrids, Oldenburg et al. (1989) mapped the HRG locus to chromosome 3. Van den Berg et al. (1990) showed that the HRG gene could be excluded from 3pter-p14. By in situ hybridization, Hennis et al. (1994) localized the HRG gene to the terminal region of 3q28-q29. They also identified a CA-repeat polymorphism in intron G and used it in linkage analysis in the CEPH families to confirm the localization to the most distal band of 3q. The availability of a PCR-based genetic polymorphism of HRG should be useful for study of the pathophysiologic role of HRG in families with thrombosis.

Shigekiyo et al. (1993) identified for the first time a family with congenital HRGP deficiency. The proband, a 43-year-old woman, suffered from right transverse sinus thrombosis during oral contraceptive treatment. Affected members of the family (5 in 3 generations) had plasma HRGP levels 25% to 35% of normal. Shigekiyo et al. (1995) could demonstrate no abnormalities in hemostatic screening tests, activities of natural anticoagulants and fibrinolytic proteins, markers of thrombin and plasmin generation, plasma levels of platelet-specific proteins, thrombin times with various concentrations of bovine thrombin, prolongation of thrombin time after addition of heparin or dermatan sulfate, and contact activation of blood coagulation in 5 'affected' members of the family. Shigekiyo et al. (1998) showed by Southern blot analysis that the proband's HRG gene carried no gross deletion or insertion. Through sequencing of all 7 exons of the HRG gene, they found a G-to-A transition at nucleotide position 429, which caused a gly85-to-glu amino acid substitution in the first cystatin-like domain. To elucidate the identified mutation as the cause of the secretion defect of HRG in the proband's plasma, they constructed and transiently expressed the recombinant Tokushima-type HRG mutant in baby hamster kidney (BHK) cells. They found that only about 20% of the Tokushima-type HRG was secreted into the culture medium, and intracellular degradation of the mutant was observed.

Souto et al. (1996) described a family with decreased levels of HRGP in 4 individuals of 3 generations. The family was brought to their attention by a woman who had developed pulmonary embolism twice at the age of 36 years. Her father had thrombosis of the central retinal artery at the age of 59 years. None of the individuals in the family was under drug therapy or affected by inflammatory diseases able to modify the plasma concentration of HRGP.

ALLELIC VARIANTS
(selected examples)

.0001 THROMBOPHELIA DUE TO HRG DEFICIENCY [HRG, GLY85GLU]

HRG TOKUSHIMA

Shigekiyo et al. (1998) demonstrated a gly85-to-glu mutation in the HRG gene in a 43-year-old Japanese woman with right transverse sinus thrombosis while taking contraceptive medication. Her plasma HRG level was only 21% of the normal.

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Interaction of Histidine-rich Glycoprotein with Human T Lymphocytes

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Histidine-rich glycoprotein (HRGP), a human plasma and platelet protein, interacts with multiple ligands *in vitro*, including heparin, plasminogen, thrombospondin, and fibrinogen/fibrin. In this study, the binding of HRGP to human T lymphocytes was characterized. The binding was specific, concentration-dependent, saturable, and reversible. Scatchard plot analysis revealed two classes of binding sites: the high affinity class had an apparent dissociation constant (K_d) of 1.92×10^{-8} M, with 0.92×10^4 sites/cell, and the low affinity class had a K_d of 4.97×10^{-7} M, with 3.7×10^4 sites/cell. HRGP binding to T cells in the presence of HRGP-depleted serum was comparable to that observed in buffer. Dot-blot analysis showed that HRGP bound to specific T cell proteins. Using both HRGP affinity chromatography and immunoprecipitation with affinity-purified anti-HRGP IgG, a major 56-kDa HRGP-binding protein in surface labeled T cell lysates was demonstrated. The 56-kDa protein was shown not to be related to the CD2 molecule on T cells. The binding characteristics of HRGP to T lymphocytes indicate a specific ligand-receptor interaction. This is the first demonstration of HRGP binding to a cell surface, and its binding to human T cells may play an important role in T lymphocyte biology.

Histidine-rich glycoprotein (HRGP)[1] is an α_2-glycoprotein present in human plasma at a substantial concentration (100–150 μg/ml) (1–3). It is also found in human platelets and is released after thrombin stimulation (4). Sequencing of the HRGP cDNA reveals a 15-nucleotide sequence encoding Gly-His-His-Pro-His which is tandemly repeated 12 times, which accounts for its basic characteristics (5). Whereas the *in vivo* role of HRGP remains to be defined, HRGP has diverse functions *in vitro*. It binds several divalent metals (6, 7), and reduces the binding of plasminogen to fibrin to retard fibrinolysis (3). It is a potent heparin-binding protein and antagonizes the anticoagulant property of heparin in plasma (8). It interacts with thrombospondin (9, 10), a multifunctional cellular adhesive protein (11–13), and becomes incorporated into fibrin clots, altering the structure of the fibrin gel (14). Recently, HRGP is found to be identical to a previously described plasma factor that inhibits rosette formation between murine T cells and erythrocytes (15, 16).

Human T lymphocytes spontaneously form rosettes with sheep erythrocytes and the sheep erythrocyte receptor has been identified as a 50–55-kDa T cell surface protein (CD2 or T11 or LFA-2 molecule) (17–20). Recent studies suggest that the CD2 molecule may represent an antigen-independent pathway of T cell activation and play an important role in T lymphocyte biology (21, 22). Despite its known inhibitory activity on T cell E-rosette formation as defined in the murine system (15, 16), the potential interaction of HRGP with human T lymphocytes and CD2 has not been explored. In this study, we characterized the specific binding of HRGP to human T cells and demonstrated a specific T cell HRGP binding protein which is not related to CD2. In the accompanying study, the functional effect of HRGP binding on T cell E-rosette formation and T cell activation was examined (32).

MATERIALS AND METHODS

Carrier-free [^{125}I]sodium iodide was purchased from Du Pont-New England Nuclear. Triton X-100, phenylmethylsulfonyl fluoride, benzamidine, and soybean trypsin inhibitor were purchased from Sigma. Paranitrophenyl paraguanidinobenzoate was purchased from ICN Biomedical Inc. Phenylalanylpropylarginyl chloromethyl ketone was purchased from Calbiochem. Heparin-Sepharose CL 6B, CNBr-activated Sepharose 4B, protein A-Sepharose 4B and a fast protein liquid chromatography system, including an anion-exchange Mono Q column, were purchased from Pharmacia LKB Biotechnology Inc. Microtitration plates and a Titertek multiscan photometer were purchased from Flow Laboratories. All reagents were of analytical grade.

Purified Proteins—HRGP was isolated from fresh platelet-poor plasma as described previously with modifications (3, 4, 23). Since HRGP was susceptible to proteolysis (16), protease inhibitors paranitrophenyl paraquanidinobenzoate 10 μM, benzamidine 1 mM, phenylmethylsulfonyl fluoride 0.4 mM, soybean trypsin inhibitor 50 mg/liter, and phenylalanylpropylarginyl chloromethyl ketone 1 μM were added in the plasma. The plasma was diluted 1:2 with distilled water and the pH-adjusted to 6.3. Batch absorption of the plasma with CM-cellulose 52 (60 g/liter plasma) was carried out at room temp for 30 min. The CM-cellulose was washed extensively with distilled water and then batch-eluted with 0.5 M NH_4HCO_3. The eluate was dialyzed overnight against 0.02 M phosphate buffer with 0.4 M NaCl, pH 6.3, and then applied to a heparin-Sepharose 6B column preequilibrated with the same buffer. After extensive washing, the column was eluted with phosphate buffer containing 1.0 M NaCl. HRGP eluted from the heparin column was >90% pure as judged by sodium dodecyl sulfate-polyacrylymide gel electrophoresis (SDS-PAGE), with M_r = 63,000 unreduced and M_r = 74,000 reduced, in agreement with published data (16, 23). The purified HRGP was free from contamination by fibrinogen, plasminogen, and antithrombin III as determined by ELISA, and functionally it bound thrombospondin and fibrinogen as demonstrated by ELISA binding assays (9, 14). Microsequencing of the purified protein revealed that the N-terminal 15 residues were identical to that published (5). In some studies, the

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[1] The abbreviations used are: HRGP, histidine-rich glycoprotein; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

purified HRGP was further applied to a Mono Q column on a fast protein liquid chromatography system and eluted with a linear NaCl gradient from 0.1 to 1.0 M NaCl as a single sharp peak at 0.25 M NaCl. Protein concentration was determined by absorbance using $E^{1\%} = 5.3$ at 280 nm (7). Antithrombin III was purified from fresh human plasma or CM-52 absorbed plasma following published methods (24, 25). Purified human fibrinogen and plasminogen were purchased from IMCO.

Human T Cells—Human peripheral blood T cells were isolated as published previously with minor modifications (26). Leukocyte-rich fractions purchased from the Stanford Medical Center Blood Bank were washed in divalent cation-free Hanks' balanced salt solution, and applied to Ficoll-Hypaque. The mononuclear cell-rich fraction was washed three times in Hanks' balanced salt solution, applied to a density percoll gradient twice, and the T cell containing fraction washed and resuspended. The purity of the T cell preparation was consistently >90% as determined by immunofluorescence using anti-CD5 (Leu-1) and anti-CD3 (OKT3) monoclonal antibodies (27).

T Cell Activation—In some studies, T cells were activated using a method described previously (27). Briefly, T cells were triggered with 12.5 ng/ml anti-CD3 (OKT3) monoclonal antibody for 40 min at 4 °C in PBS. Cells were then cultured at 37 °C at 10^6 cells/ml in Iscove's modified Dulbecco's medium containing 15% fetal calf serum, 5×10^{-4} M mercaptoethanol, 1% glutamine, antibiotics, and 100 units/ml recombinant IL2 (Cetus Corp., specific activity 6.0×10^9 units/g protein). At time 0 and after 3–6 days, cell aliquots were stained with OKT3-fluorescein isothiocyanate and p55 IL2R 2A3-phycoerythrin antibodies (Becton Dickinson) and analyzed with a two-laser flow cytometer (Ortho flurograph 2150). In a typical experiment, ≈44 and 72% of cultured cells expressed IL2 receptors after 3 and 6 days of incubation, respectively.

Radioisotope Labeling—Purified HRGP was labeled with ^{125}I using the Iodobead method (Bio-Rad) following the manufacturer's instructions. The radiolabeled HRGP was repurified by heparin-Sepharose affinity chromatography, with specific radioactivity in the range of 400–500 cpm/ng HRGP. The labeled protein samples were precentrifuged at $14,000 \times g$ for 3 min to remove potential aggregates before each epxeriment.

HRGP Binding to Human T Cells— T cells (3×10^7 cells/ml) were incubated in 20 mM phosphate-buffered saline, pH 7.4, containing 2% bovine serum albumin (PBS/BSA) for 1 h at 4 °C. The cells were aliquoted and ^{125}I-HRGP, in the presence or absence of 50-fold excess of unlabeled HRGP, was added and incubated for 1 h at 4 °C. The final volume of the reaction mixture was 300 μl with a cell concentration of 1×10^7 cells/ml. At the end of incubation, cell-bound HRGP was separated from unbound free HRGP by centrifuging the cell suspensions through silicone oil (6.7 parts Dow Corning oil 550, 3.3 parts Dow Corning oil 556, specific gravity 1.040) at $14,000 \times g$ for 10 min in a Beckman Microfuge. Tips of the Eppendorf tubes containing the cell pellets were excised and cell-bound radioactivity determined in a γ counter. Each experiment was repeated at least three times with each data point done in duplicate. In some studies, T cells were pretreated with heparinase (5 units/ml) in 0.1 M NaCl, 0.5 M sodium acetate, pH 6.5, for 30 min prior to HRGP binding. For control studies, human or sheep red blood cells were isolated from citrated whole blood by differential centrifugations, and washed with PBS/BSA three times before HRGP binding. The human monocytoid cell line U937 was obtained from Dr. Alan Krensky, and the Burkitt cell line and the Epstein-Barr virus transformed B cell lines were obtained from Dr. Stephen Smith, Stanford University Medical School.

Immunodepletion of HRGP from Normal Serum—Anti-HRGP IgG was isolated from immune rabbit serum and immunopurified using HRGP-Sepharose as described previously (4). To immunodeplete HRGP from normal serum, immunopurified anti-HRGP IgG was coupled to CNBr-activated Sepharose 4B at 3.7 mg IgG/ml of packed gel. Ten ml of normal serum was absorbed with 1 ml of IgG-coupled Sepharose 4B for 1 h at room temperature for three times. HRGP concentration was determined by a double antibody ELISA (4).

HRGP Binding to T Cell Lysates by Dot-blot Analysis—Cell lysates were dotted on nitrocellulose membranes and air-dried. Nonspecific binding sites on the nitrocellulose membranes were blocked by incubation in 0.02 M Tris-HCl buffer, pH 7.4, containing 0.05% Tween 20 and 5% non-fat dry milk for 1 h at room temperature. ^{125}I-HRGP in the same buffer was added for 1 h at room temp. The membranes were extensively washed, air-dried, and processed for autoradiography.

HRGP Affinity Chromatography of Surface-iodinated T Cell Lysates—15 mg of purified HRGP was coupled to 4 ml of CNBr-activated Sepharose 4B. Isolated human T cells were surface-iodinated using the immobilized lactoperoxidase bead method (Bio-Rad) following the manufacturer's instructions. The labeled cells were washed with phosphate buffer, pH 7.4, three times and solubilized by 1% Triton X-100 in the same buffer with protease inhibitors at 4 °C for 1 h. After centrifugation to remove any insoluble materials, aliquots of the cell lysates were applied to a HRGP-Sepharose 6B column and eluted with 0.1 M glycine, pH 2.5, after extensive washings. The eluates were analyzed by SDS-PAGE and autoradiography.

Immunoprecipitation of HRGP-binding Protein from T Cell Lysates—Triton X-100-solubilized surface-labeled T cell lysates were incubated with HRGP (10–100 μg/ml) for 1 h at 4 °C. Affinity-purified anti-HRGP IgG was added and incubated overnight at 4 °C. The immune complexes were adsorbed with protein A-Sepharose and after extensive washings, boiled with sample buffer and analyzed by SDS-PAGE.

RESULTS

HRGP Binding to Human T Cells—^{125}I-HRGP bound to isolated human T cells in a concentration-dependent and saturable manner (Fig. 1). Nonspecific binding, as defined by binding in the presence of 50-fold molar excess of unlabeled HRGP, was ≈20% of total binding. The binding reached a steady state at 30 min at 4 °C and was reversible, with 70% of the surface bound HRGP displaced by 50-fold molar excess of unlabeled HRGP (Fig. 2). Scatchard plot of the specific HRGP-binding isotherm was curvilinear, suggesting either two or more classes of HRGP binding sites or a single class of binding sites with negative cooperativity (Fig. 3). Assuming the presence of two independent classes of binding sites, the high affinity class had an apparent dissociation constant (K_d) of 1.92×10^{-8} M, with $\approx 0.92 \times 10^4$ sites/cell, and the low affinity class had a K_d of 4.97×10^{-7} M, with $\approx 3.7 \times 10^4$ sites/cell. Two mM EDTA had no effect on the HRGP binding to T cells, suggesting that the binding was not divalent cation dependent. Since HRGP binds to zinc with high affinity (6, 7), the effect of $ZnCl_2$ was tested. Two μM $ZnCl_2$ had no significant effect on HRGP binding to T cells.

Specificity of HRGP Binding to T Cells—To determine the specificity of HRGP binding, binding of ^{125}I-HRGP to T cells was performed in the presence of molar excesses of unlabeled ligands (Fig. 4). Unlabeled HRGP competitively inhibited the binding of ^{125}I-HRGP, with maximal effect achieved at 10-

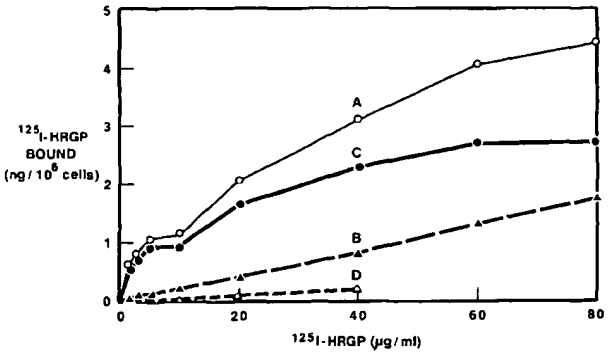

FIG. 1. **HRGP binding to human T lymphocytes.** Isolated human T cells and washed human or sheep red blood cells were incubated in PBS/BSA for 1 h at 4 °C. ^{125}I-HRGP was added, in the presence or absence of 50-fold excess unlabeled HRGP, and incubated for 1 h at 4 °C. The cell suspensions were spun through silicone oil, and the amount of radioactivity in the cell pellets counted. Each point is the mean of duplicate determinations. *A*, ^{125}I-HRGP binding in the absence of unlabeled HRGP (total binding). *B*, ^{125}I-HRGP binding in the presence of 50-fold excess of unlabeled HRGP (nonspecific binding). *C*, specific binding of ^{125}I-HRGP (total minus nonspecific binding). *D*, ^{125}I-HRGP binding to washed sheep or human red blood cells in the absence of unlabeled HRGP.

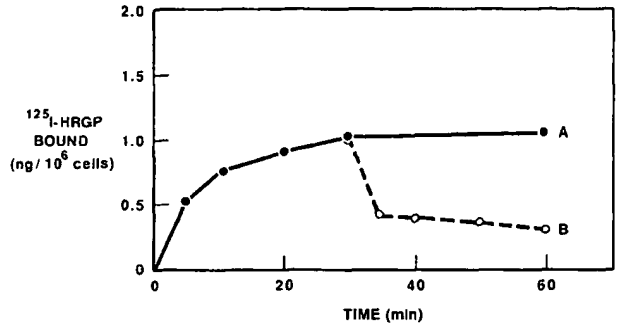

FIG. 2. **Time course and reversibility of HRGP binding to T lymphocytes.** T lymphocytes were incubated in PBS/BSA for 1 h at 4 °C. ^{125}I-HRGP (final concentration 10 μg/ml) was added. Duplicate aliquots of the cell suspensions were removed at various time points and cell-bound radioactivity determined (*A*). After 30-min incubation with radiolabeled HRGP, 50-fold molar excess of unlabeled HRGP was added and cell-bound radioactivity at different time points determined (*B*).

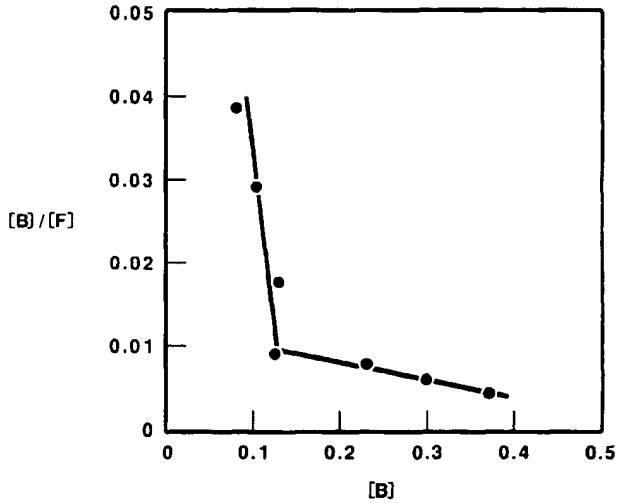

FIG. 3. **Scatchard plot analysis of HRGP binding to T lymphocytes.** The specific HRGP binding to T cells (Fig. 1*C*) was analyzed. The *lines* represent linear regression by least squares analysis of data points, $y = 0.00794–0.052 \times$, $r = -0.963$ (high affinity); $y = 0.00123–0.002 \times$, $r = -0.983$ (low affinity).

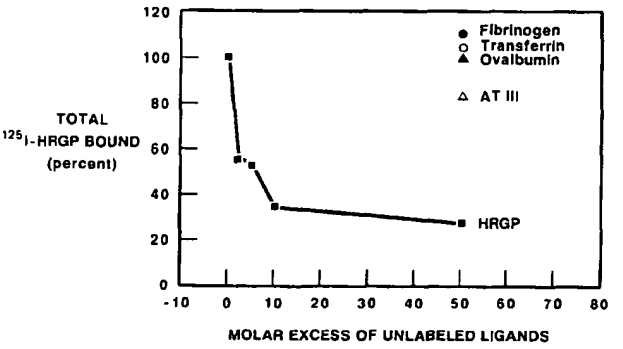

FIG. 4. **Binding of ^{125}I-HRGP at molar excess of unlabeled ligands.** Binding studies of labeled HRGP, in the presence of molar excesses of unlabeled ligands, were performed as described in Fig. 1.

fold molar excess, whereas transferrin, ovalbumin, fibrinogen, and antithrombin III had no significant effect at 50-fold molar excess.

The binding of HRGP to T cells was also compared to that of other cell types. HRGP binding to human red cells, Burkitt cells (a human B cell line), and COS-7 cells (a monkey kidney line) was minimal, indicating the relative specificity of HRGP binding to T cells (Table I). Although there was significant HRGP binding to U937 cells (a human monocytoid cell line) and Epstein-Barr virus-transformed human B cells (Table I), heparin effectively abolished HRGP binding to both cell types, suggesting that binding was mediated by cell surface heparin-like proteoglycans (8). In contrast, heparin did not significantly interfere with HRGP binding to T cells. Pretreatment of T cells with heparinase also had no effect on subsequent ^{125}I-HRGP binding. Taken together, the data indicate that HRGP binding to human T cells is relatively specific and is mediated by distinct cell surface proteins not related to proteoglycans.

To demonstrate that the binding of ^{125}I-HRGP was not due to a labeling artifact, binding was performed with various ratios of labeled to unlabeled HRGP while maintaining the total HRGP concentration constant (Fig. 5). The percentage of ^{125}I-HRGP in the bound HRGP correlated in a linear fashion with the percentage of ^{125}I-HRGP in the total HRGP applied (correlation coefficient = 0.99), indicating that the labeled and unlabeled HRGP had similar dissociation constants.

^{125}I-HRGP binding to T cells were carried out at two concentrations to characterize the cell-bound HRGP at the high affinity and the low affinity binding sites. SDS-PAGE

TABLE I

HRGP binding to different cell types

Cells were washed with PBS three times and then incubated with PBS/BSA for 1 h. Binding with ^{125}I-HRGP (40 μg/ml) was carried out at 4 °C for 1 h in PBS/BSA, in the presence or absence of 50 × molar excess of heparin. Cell-bound radioactivity was then determined. Data represent the mean ± S. D. ($n = 4$). ND, not determined. EBV, Epstein-Barr virus.

Cell types	HRGP bound	
	−Heparin	+Heparin
	ng/10^6 cells	
T cells	2.0 ± 0.27	1.64 ± 0.32
U937 cells	1.87 ± 0.21	0.016 ± 0.022
EBV-B cells	1.36 ± 0.43	0.022 ± 0.031
Burkitt cells	0.035 ± 0.025	ND
COS-7 cells	0.012 ± 0.023	ND
Red blood cells	0.022 ± 0.014	ND

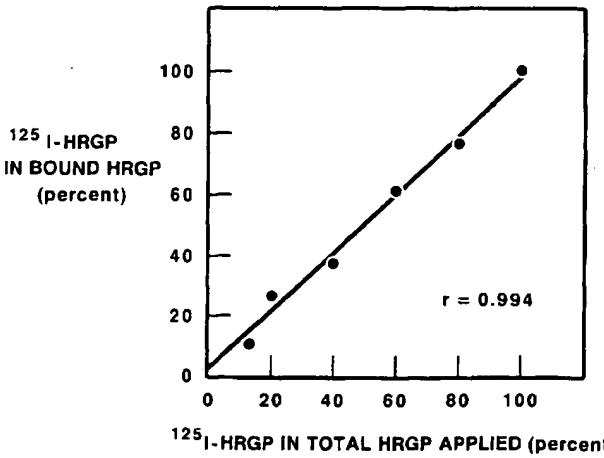

FIG. 5. **Specific binding of ^{125}I-HRGP at various ratios of ^{125}I-HRGP to unlabeled HRGP.** HRGP binding was performed as described in Fig. 1. The total concentration of HRGP (labeled plus unlabeled) was kept constant at 10 μg/ml. $y = 0.96 \times + 1.19$, $r = 0.99$.

analyses revealed no major differences between the ^{125}I-HRGP applied and the cell-bound ^{125}I-HRGP at the two different ligand concentrations (Fig. 6).

HRGP Binding to T Cells in HRGP-depleted Serum—To determine if HRGP would bind to T cells in a physiologic milieu, normal human serum was immunodepleted of HRGP by anti-HRGP IgG-coupled Sepharose 4B. HRGP concentration in the immunodepleted serum was ≤5 μg/ml as determined by ELISA (4). Binding of ^{125}I-HRGP to T cells in the presence of HRGP-depleted serum was comparable to that obtained in buffer, indicating that serum proteins did not have a significant modulating effect on HRGP binding (Fig. 7).

HRGP Affinity Chromatography of Surface-iodinated T Cell Lysates—^{125}I-HRGP bound to human T cell lysates but not red blood cell lysates on a dot-blot analysis, indicating that HRGP bound to specific T cell proteins (data not shown). Purified plasminogen and albumin served as positive and negative controls, respectively, in this experiment. To characterize the specific HRGP-binding protein(s) on the T cell surface, surface-iodinated T cell lysates were applied to a HRGP-Sepharose affinity column. The column eluate was analyzed by SDS-PAGE and autoradiography and revealed a major 56-kDa HRGP-binding protein and minor bands with molecular masses of 34, 31, and 28 kDa (Fig. 8). Control study using an albumin-Sepharose column was negative. Furthermore, the intensity of the 56-kDa band was markedly reduced when the labeled T cell lysate was applied in the presence of 50-fold excess of unlabeled T cell lysate, indicating that it was not related to a labeling artifact.

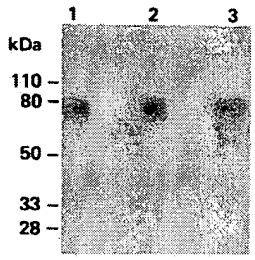

FIG. 6. **SDS-PAGE analyses of cell-bound ^{125}I-HRGP.** ^{125}I-HRGP binding to T cells was carried out at two concentrations (4 μg/ml and 30 μg/ml). Cell-bound ^{125}I-HRGP was extracted by boiling the washed cell pellets in SDS sample buffer and analyzed by SDS-PAGE. *Lane 1* represents input ^{125}I-HRGP. *Lanes 2* and *3* represent cell-bound ^{125}I-HRGP at 4 μg/ml and 30 μg/ml ligand concentrations, respectively.

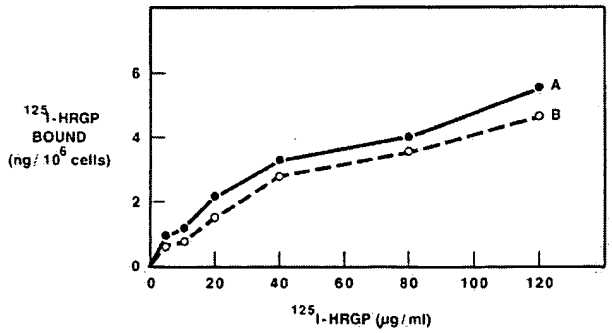

FIG. 7. **Specific binding of ^{125}I-HRGP in the presence of HRGP-depleted serum.** Normal serum was immunodepleted of HRGP as described under "Materials and Methods." T cells were resuspended in PBS/BSA or undiluted immunodepleted serum, and HRGP binding was performed as described in Fig. 1. *A*, ^{125}I-HRGP binding in the presence of PBS-BSA; *B*, ^{125}I-HRGP binding in the presence of HRGP-depleted serum.

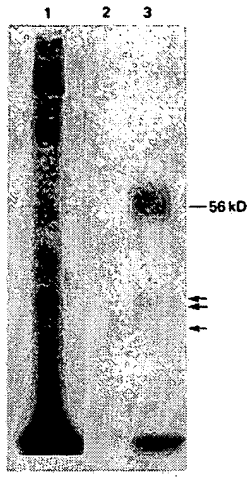

FIG. 8. **HRGP affinity chromatography of surface-iodinated T cell lysates.** Aliquots of Triton X-100-solubilized surface-labeled T cell lysates were applied to HRGP-Sepharose and albumin-Sepharose columns and eluted with 0.1 M glycine, pH 2.5, after extensive washings. The eluates were analyzed by SDS-PAGE and autoradiography. *Lane 1*, total T cell lysates; *lane 2*, albumin-Sepharose column eluate; and *lane 3*, HRGP-Sepharose column eluate.

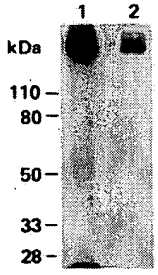

FIG. 9. **Immunoprecipitation of HRGP-binding protein from T cell lysates.** Triton X-100-solubilized surface-labeled T cell lysates were incubated with HRGP (*lane 1*) or control ovalbumin (*lane 2*), followed by the sequential additions of affinity-purified anti-HRGP IgG and protein A-Sepharose. After extensive washings, the immunoprecipitates were extracted and analyzed by SDS-PAGE and autoradiography.

Immunoprecipitation of HRGP-binding Protein from T Cell Lysates—As an alternative approach to characterize the T cell HRGP-binding protein, immunoprecipitation studies of mixtures of surface-labeled T cell lysates and HRGP by affinity-purified anti-HRGP IgG were done. SDS-PAGE of the immunoprecipitates revealed a 56-kDa T cell surface-labeled protein (Fig. 9), similar to the one observed in the HRGP affinity chromatography studies. The 210-kDa T cell surface protein found in the HRGP immunoprecipitate was nonspecific and was also observed in the control study with ovalbumin. Further studies showed that it was apparently a protein A binding protein on the T cell surface.

Relationship of the HRGP-binding Protein to CD2—We have recently obtained evidence demonstrating that HRGP interferes with human T cell rosette formation with sheep erythrocytes (32), consistent with previous observations made in the murine system (15, 16). Since sheep erythrocyte binding to human T cells is mediated by CD2, a 50–55-kDa surface protein with 40,000–50,000 molecules/cell (21), we investigated whether the 56-kDa HRGP-binding protein was related to CD2. A number of approaches were taken: (i) T cells were activated and demonstrated to have an approximately 3-fold increase in CD2 expression as determined by ^{125}I-anti-CD2 (Leu-5b) binding, in agreement with published results (22).

However, binding of ^{125}I-HRGP to activated T cells was similar to that of resting T cells and did not correlate with the increased CD2 expression. (ii) Anti-CD2 (Leu-5b) did not interfere with the specific ^{125}I-HRGP binding to T cells; alternatively, HRGP did not affect ^{125}I-anti-CD2 (Leu-5b) binding to T cells. (iii) The 56-kDa T cell HRGP-binding protein identified in the HRGP affinity chromatography of labeled T cell lysates was not immunoprecipitated by Leu-5b. Alternatively, immunoprecipitation of a mixture of ^{125}I-HRGP and T cell lysates with Leu-5b did not reveal the presence of ^{125}I-HRGP in the precipitate. Under identical conditions, Leu-5b immunoprecipitated CD2 from surface labeled T cell lysates. (iv) Surface-labeled T cell lysate was "precleared" of its HRGP-binding protein by incubation with HRGP-Sepharose beads and then subjected to immunoprecipitation with Leu-5b. Leu-5b successfully immunoprecipitated CD2 from the precleared T cell lysate, and the intensity of the immunoprecipitate on the autoradiogram was not different from that of noncleared T cell lysate. Taken together, the data suggest that the 56-kDa HRGP-binding protein is not related to CD2 and its identity remains to be determined.

DISCUSSION

In this study, the binding of HRGP to human T lymphocytes was characterized. The binding was specific, concentration-dependent, saturable, and reversible (Figs. 1, 2, and 4–6), suggesting that HRGP binding was mediated by specific receptor(s) on the T cell surface. This was supported by the Scatchard plot analysis demonstrating the presence of discrete cellular HRGP-binding sites (Fig. 3). Using HRGP affinity chromatography and immunoprecipitation with affinity-purified anti-HRGP, a major 56-kDa T cell surface HRGP-binding protein was demonstrated (Figs. 8 and 9). Whether the 56-kDa HRGP-binding protein represents the cellular HRGP receptor remains to be determined.

Since both T cells and HRGP may interact with multiple plasma proteins *in vivo*, it is important to examine whether the binding of HRGP to T cells can occur in a more physiologic milieu. HRGP binding in the presence of HRGP-depleted serum was comparable to that observed in a buffer system (Fig. 7). Of interest, both fibrinogen and heparin, which have been previously shown to interact with HRGP (8, 14), also did not interfere with HRGP binding to T cells, suggesting that the T cell-binding site and the fibrinogen- and heparin-binding sites are located in different parts of the HRGP molecule.

The functional significance of HRGP binding to human T cells is an important issue that needs to be defined. Recently, we have obtained data in support of a functional role for HRGP binding to T cells. HRGP partially interfered with human T cell rosette formation with sheep erythrocytes and modulated interleukin-2 receptor expression and interferon-γ production by activated T cells, suggesting that HRGP may play an important role in modulating T lymphocyte activation (32). The T cell sheep erythrocyte receptor plays a major role in T cell activation (21, 22, 28–30) and has been identified as the CD2 (T11 or LFA-2) molecule, a 50–55-kDa surface protein with 40,000–50,000 molecules/cell (21), in the same range as the number of HRGP-binding sites. We therefore examined whether the 56-kDa HRGP-binding protein is related to CD2. However, using a variety of approaches, we showed that the 56-kDa HRGP-binding protein on the T cell surface is not related to the CD2 molecule, and its exact identity is under active investigation in our laboratories. Our data suggest that HRGP at physiologic concentrations binds to T cells, at a site distinct from CD2, and modulates T cell activation and immunoregulation. It is of interest that a T cell surface molecule different from CD2 has recently been reported to be involved in the spontaneous rosette formation with erythrocytes (31).

HRGP interacts with multiple ligands *in vitro*, including divalent cations, plasminogen, heparin, thrombospondin, and fibrinogen/fibrin (3, 6, 9, 10, 14), and may modulate thrombotic and inflammatory events (3, 9, 14). Our study is the first demonstration of the specific binding of this protein to T cells and raises the possibility that some of the HRGP-ligand interactions observed *in vitro* may take place on the lymphocyte cell surface and play a role in T lymphocyte biology.

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Heparin Binding Properties of Human Histidine-rich Glycoprotein

MECHANISM AND ROLE IN THE NEUTRALIZATION OF HEPARIN IN PLASMA*

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Human histidine-rich glycoprotein was found to interact strongly with heparin both in purified systems and in plasma, resulting in neutralization of the anticoagulant activity of heparin. In purified systems, histidine-rich glycoprotein and heparin react with apparent 1:1 stoichiometry to form a complex with a dissociation constant of 7 nM. Covalent heparin-antithrombin complex still reacts with histidine-rich glycoprotein to form a complex with a dissociation constant of 29 nM. The interaction between a M_r = 4300-heparin fragment and histidine-rich glycoprotein appeared to be more complex.

The mechanism of the interaction between histidine-rich glycoprotein and heparin appeared to be different from that between antithrombin III and heparin, since the former is abolished by EDTA and occurs both with heparin molecules having a high affinity or a low affinity for antithrombin III.

In plasma, histidine-rich glycoprotein efficiently counteracts the anticoagulant activity of heparin. Both the thrombin times and the activated factor X inhibition following addition of heparin are markedly prolonged in the absence of histidine-rich glycoprotein and shortened by addition of purified histidine-rich glycoprotein.

Low affinity heparin was found to efficiently compete with high affinity heparin for binding to histidine-rich glycoprotein but not to antithrombin III. This results in an increased anticoagulant activity of high affinity heparin in the presence of low affinity heparin. Since the effect of histidine-rich glycoprotein on the anticoagulant properties of heparin is clearly demonstrated in normal plasma, it may be of clinical significance.

Heparin binds to antithrombin III and the heparin-antithrombin III complex inhibits a number of activated coagulation factors, including thrombin, factor IXa, factor Xa, factor XIa, and factor XIIa (1). The half-life of the anticoagulant activity of heparin in plasma following intravenous injection in man varies between 23 min and 2.5 h (2), whereas that of antithrombin III is 2.7 days (3). The main mechanism of disappearance of the anticoagulant activity appears to be by removal of free heparin and dissociation of the heparin-antithrombin III complex and not by clearing of the intact complex (4).

In plasma only a fraction of the heparin specifically binds to and activates antithrombin III (5, 6). Heparin interacts with albumin (7), fibrinogen (7), fibronectin (8), lipoprotein lipase (9), and with α_1-acid glycoprotein (10). Platelet factor 4 neutralizes the anticoagulant activity of heparin (11, 12) by interaction with multiple binding sites on the polysaccharide (13). The physiological importance of these interactions has, however, not been established.

Clinical grade heparin preparations are very heterogeneous with respect to molecular size, chemical structure, and anticoagulant activity. Molecular weights range between 6,000 and 30,000 (14). Heparin can be fractionated into a component with high affinity for antithrombin III and high anticoagulant activity, and a low affinity fraction with low anticoagulant activity (5, 6, 15). Heparin fractions of decreasing molecular weight show a progressive loss of antithrombin activity but not of antifactor Xa activity (16, 17). A minimal sequence of eight monosaccharide units in the heparin molecule is required for binding to antithrombin III (18).

In the present study we report that histidine-rich glycoprotein (19) has a strong affinity for heparin and plays an important role in the neutralization of heparin in human plasma. Histidine-rich glycoprotein was first isolated as a glycoprotein with a M_r of 60,000 (19, 20), but later this form appeared to be a proteolytic derivative of a molecule with M_r = 75,000 (20). It has been shown to interact with heparin (19), with the main lysine-binding site of plasminogen (21), with divalent cations (22), and with the rosette formation between erythrocytes and lymphocytes (23).

EXPERIMENTAL PROCEDURES

Proteins and Reagents—Histidine-rich glycoprotein was purified from human plasma by chromatography on the insolubilized high affinity lysine-binding site of plasminogen followed by DEAE-Sephadex and immunoadsorption chromatography (21). When this procedure is carried out as quickly as possible, at 4 °C or in the presence of aprotinin, proteolytic degradation of histidine-rich glycoprotein is avoided and a molecule with M_r of approximately 75,000 is obtained. If no precautions are taken, the degraded form with M_r = 60,000 is obtained. The present study was carried out with preparations containing only native histidine-rich glycoprotein as evidenced by polyacrylamide gel electrophoresis. Protein concentration was determined by the method of Lowry using bovine albumin as a standard (24). Plasma depleted in histidine-rich glycoprotein was obtained by adsorption on Cm-cellulose (19); the residual concentration of histidine-rich glycoprotein in this plasma, as measured by Laurell electroimmunoassay (25) using a homemade monospecific rabbit antiserum, was less than 5%, whereas the antithrombin III and the fibrinogen levels were normal.

Antithrombin III was purified from human plasma by chromatography on heparin-Ultrogel (26). Clinical grade heparin was obtained from Roche (Brussels, Belgium); the high affinity and the low affinity fraction were separated by affinity chromatography on antithrombin III (15). The heparin fragment with M_r = 4,300 was obtained by partial degradation of intact heparin with nitrous acid (27); it was a kind gift from KabiVitrum (Stockholm, Sweden, courtesy of E. Holmer). The covalent complex between high affinity heparin (M_r = 15,000) and antithrombin III was prepared as previously described

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(4). Factor Xa[1] and the substrate S-2222 were obtained from Kabi-Vitrum (Amsterdam, The Netherlands); the concentration of factor Xa was determined by titration against antithrombin III of known concentration. Thrombin used for thrombin time assays was Toposstasine (Roche, Brussels, Belgium).

Neutralization of Heparin Activity by Histidine-rich Glycoprotein in a Purified System—The influence of histidine-rich glycoprotein on the inhibition of factor Xa by antithrombin III in the presence of high affinity heparin or of the M_r = 4,300 fragment of heparin and by a covalent heparin-antithrombin III complex was investigated in the presence of the synthetic substrate S-2222 (28).

A calibration curve was constructed by measuring the inhibition of factor Xa (1.2 nM final concentration) by antithrombin III (107 nM final concentration) in the presence of S-2222 (0.2 mM final concentration) and different concentrations of high affinity heparin (0–10 nM final concentration) or of a M_r = 4,300 heparin fragment (0–32 nM final concentration) in a total volume of 1 ml in 0.1 M Tris-HCl buffer, pH 7.60. The disappearance rate of the amidolytic activity was continuously recorded at 405 nm on a Unicam SP 1800 Spectrophotometer for 3 to 8 min. At different time intervals, residual enzyme activity was determined from the slope of the curve (before 50% of the enzyme was inactivated) and exponentially plotted *versus* reaction time. Apparent rate constants were calculated using the equation, $k_{app} = (\ln 2/t_{1/2}) \times (1 + [S^0]/K_m)$ and plotted against the heparin concentration. Indeed, the inhibition of Xa by antithrombin III (A) in the presence of heparin (H) and the synthetic substrate S-2222 (S), is described by the following equilibria and reactions:

$$\begin{array}{l} \mathrm{Xa} + \mathrm{A{\cdot}H} \overset{K_i}{\rightleftarrows} \mathrm{Xa{\cdot}A{\cdot}H} \overset{k_2}{\rightarrow} \mathrm{Xa{\cdot}A} + \mathrm{H} \\ + \\ \mathrm{S} \\ \Updownarrow K_m \\ \mathrm{Xa{\cdot}S} \end{array}$$

with K_i, dissociation constant of Xa·A·H complex; k_2, first order rate constant of irreversible inactivation; K_m, Michaelis constant for S-2222 (0.3 mM); and by the following rate equation:

$$v = \frac{d[\mathrm{Xa}]}{dt} + \frac{d[\mathrm{Xa{\cdot}S}]}{dt} = -k_2[\mathrm{Xa{\cdot}A{\cdot}H}] = -\frac{k_2}{K_i}[\mathrm{A{\cdot}H}]{\cdot}[\mathrm{Xa}]$$

which yields upon transformation:

$$\ln [\mathrm{Xa}] = -\frac{k_2/K_i}{1 + [S^0]/K_m}[\mathrm{A{\cdot}H}]t$$

with k_2/K_i, the second order rate constant of factor Xa inactivation; [A·H], the initial concentration of antithrombin III-heparin complex; t, reaction time.

The slope of the plot of ln [Xa] *versus* t represents the apparent rate constant of the inhibition of Xa by A·H in the presence of S (k'_{app}) and is determined from the half-life ($t_{1/2}$) as $k'_{app} = \frac{\ln 2}{t_{1/2}}$ and corresponds to $k'_{app} = -\frac{k_2/K_i}{1 + [S^0]/K_m} \cdot [\mathrm{A{\cdot}H}]$. The apparent rate constant of the inhibition of Xa by A·H in the absence of S (k_{app}) then corresponds to $k_{app} = -\frac{k_2}{K_i}[\mathrm{A{\cdot}H}] = k'_{app}\left(1 + \frac{[S^0]}{K_m}\right)$. The second order rate constant of the inhibition of Xa by A·H then may be obtained as $-\frac{k_2}{K_i} = \frac{k_{app}}{[\mathrm{A{\cdot}H}]} = \frac{k'_{app}}{[\mathrm{A{\cdot}H}]}\left(1 + \frac{[S^0]}{K_m}\right)$.

The influence of histidine-rich glycoprotein on the inhibition of factor Xa by antithrombin III in the presence of high affinity heparin or of a M_r = 4,300 heparin fragment was then studied in a similar system. Therefore, antithrombin III (107 nM final concentration), S-2222 (0.2 mM final concentration), and high affinity heparin (7.3 nM final concentration) or M_r = 4,300 heparin (32 nM final concentration) were incubated at 37 °C for 2 min with different concentrations of histidine-rich glycoprotein (4–60 nM for the experiments with high affinity heparin and 5–200 nM for the measurements with the M_r = 4,300 heparin). Factor Xa was then added (1.2 nM final concentration) and the disappearance rate of the amidolytic activity was continuously recorded. Under these conditions, heparin and the M_r = 4,300 heparin are in excess of factor Xa and pseudo-first order conditions apply as evidenced by linear plots of the logarithm of residual factor Xa concentration *versus* time.

For each concentration of histidine-rich glycoprotein the apparent rate constant was calculated from the $t_{1/2}$ as described above. Addition of histidine-rich glycoprotein results in a decrease of the rate of inhibition of factor Xa by antithrombin III in the presence of heparin, due to reaction with and neutralization of heparin. The residual free heparin concentration is determined from the apparent rate constant in the presence of histidine-rich glycoprotein, using calibration curves of the apparent rate constant (k_{app}) of the inhibition of factor Xa by antithrombin III *versus* the heparin concentration. This residual free heparin concentration, further designated as apparent heparin concentration and expressed in per cent of the total concentration, is then plotted against the residual free histidine-rich glycoprotein concentration. The concentration of free histidine-rich glycoprotein itself is determined from the total amount minus that bound to heparin, assuming a 1:1 interaction between heparin and histidine-rich glycoprotein. This assumption appears to be valid for intact heparin but not for the M_r = 4,300 fragment (see "Results").

The influence of low affinity heparin on the inhibition of high affinity heparin by histidine-rich glycoprotein was studied in a similar system. Therefore histidine-rich glycoprotein (30 nM final concentration), antithrombin III (107 nM final concentration), high affinity heparin (7.3 nM final concentration) and S-2222 (0.2 mM final concentration) were incubated at 37 °C for 2 min with different concentrations of low affinity heparin (0–10 nM final concentration). Factor Xa (1.2 nM final concentration) was then added and the residual factor Xa activity continuously recorded. Apparent rate constants were then determined for each concentration of low affinity heparin.

The influence of histidine-rich glycoprotein (0–80 nM final concentration) on the inhibition of factor Xa (1.2 nM final concentration) by a covalent heparin-antithrombin III complex (10 nM final concentration) was studied in a similar way. The apparent rate constants were plotted against the total concentration of histidine-rich glycoprotein. The dissociation constant of the interaction between histidine-rich glycoprotein and the covalent heparin-antithrombin III complex was determined as the concentration of histidine-rich glycoprotein that decreased the apparent rate constant to 50%.

Neutralization of Heparin Activity by Histidine-rich Glycoprotein in Human Plasma—The influence of histidine-rich glycoprotein on the neutralization of heparin in plasma was evaluated by determination of thrombin times of normal plasma, plasma depleted in histidine-rich glycoprotein, and the depleted plasma reconstituted with purified histidine-rich glycoprotein. The thrombin times were recorded as the clotting time in mixtures of 0.2 ml of plasma (diluted ½ with water) and 0.1 ml of thrombin solution (approximately 0.2 NIH units/ml final concentration) to which clinical grade heparin (final concentrations, 0–1.0 IU/ml of plasma) or high affinity heparin (final concentrations, 0–0.6 IU/ml of plasma) was added. In addition, the combined effect of low affinity heparin and high affinity heparin on the thrombin time of normal plasma was investigated. Therefore, the plasma was incubated during 30 s with high affinity heparin (40 nM final concentration) and increasing concentrations of low affinity heparin (0–800 nM final concentration). Thrombin times reported always represent mean values of at least two separate measurements.

Alternatively, the influence of histidine-rich glycoprotein on the neutralization of heparin in plasma was studied by measurements of the inhibition of factor Xa. A calibration curve was constructed by measuring the factor Xa inhibition in plasma depleted in histidine-rich glycoprotein, following addition of different amounts of clinical grade heparin or of a heparin fragment with M_r = 4,300. Therefore, 50 μl of depleted plasma was incubated with heparin (0–0.1 IU/ml final concentration) and with factor Xa (8.3 nM final concentration) in a total volume of 300 μl in 0.1 M Tris-HCl buffer, pH 7.60. After incubation at 37 °C for 30 s, residual factor Xa was measured following addition of 700 μl of a mixture of S-2222 (0.4 mM final concentration) and protamine (6 IU/ml final concentration) in 0.05 M Tris-HCl buffer, pH 8.40, containing 7.5 mM EDTA. The change in absorbance at 405 nm, which is proportional to the residual factor Xa activity was then plotted against the heparin concentration. Upon addition of a known amount of heparin (0.08 IU/ml for clinical grade and 0.04 IU/ml for the M_r = 4,300 heparin) the residual factor Xa activity was then measured in the same way in the depleted plasma, which was reconstituted with purified histidine-rich glycoprotein to different concentrations. The apparent heparin concentration in the plasmas was then read from the calibration curves and expressed as a percentage of the amount added.

[1] The abbreviations used are: Xa, activated factor X; S-2222; *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide·hydrochloride; HRG, histidine-rich glycoprotein.

Study of the Mechanism of the Interaction between Histidine-rich Glycoprotein and Heparin—The interaction between heparin and purified histidine-rich glycoprotein was studied by crossed immunoelectrophoresis using a monospecific antiserum to histidine-rich glycoprotein. Crossed immunoelectrophoresis was carried out in the absence and in the presence of clinical grade heparin (50 IU or 0.5 mg/ml) or low affinity heparin (0.2 mg/ml) added to the agarose gel. In addition, the influence of EDTA (50 μM final concentration) was investigated on the mobility of histidine-rich glycoprotein in the presence and in the absence of heparin. For comparison, the same experiments were performed with purified antithrombin III and a monospecific antiserum against antithrombin III.

The influence of EDTA and of divalent metal ions on the interaction between heparin and histidine-rich glycoprotein was further investigated in a kinetic system. Therefore, antithrombin III (107 nM final concentration), histidine-rich glycoprotein (30 nM final concentration), high affinity heparin (7.3 nM final concentration) and S-2222 (0.2 mM final concentration) were incubated at 37 °C for 2 min with different concentrations of EDTA (0–10 μM final concentration). Factor Xa was then added (1.2 nM final concentration) and the disappearance rate of the amidolytic activity in 0.1 M Tris-HCl buffer, pH 7.6, was continuously recorded. Alternatively, the histidine-rich glycoprotein (1 μM in 0.1 M Tris-HCl buffer, pH 7.60) was treated with EDTA (10 mM final concentration) and the EDTA was subsequently removed by dialysis. The influence of the addition of different divalent cations (10 μM final concentration) to the incubation mixture was then investigated using the same kinetic system, after treatment of all buffers and solutions with Chelex 100.

RESULTS

Neutralization of Heparin Activity by Histidine-rich Glycoprotein in a Purified System

Determination of the Dissociation Constants of the Complexes between Histidine-rich Glycoprotein and High Affinity Heparin, M_r = 4,300 Heparin and a Covalent Heparin-Antithrombin III Complex—The apparent rate constant of the inhibition of factor Xa by antithrombin III increases linearly with the heparin concentration in the concentration range from 0–10 nM for high affinity heparin and 0–32 nM for the M_r = 4,300 heparin. Addition of histidine-rich glycoprotein induces a concentration-dependent reduction of the inhibition rate constant of factor Xa by antithrombin III in the presence of heparin. Control experiments in the same system revealed no inhibition of factor Xa by histidine-rich glycoprotein alone or in the presence of heparin nor an influence of histidine-rich glycoprotein on the inhibition of factor Xa by antithrombin III alone. These findings indicate that the histidine-rich glycoprotein efficiently competes with antithrombin III for binding of heparin, thereby neutralizing the heparin activity and thus decreasing the inhibition rate of factor Xa. When the apparent rate constants measured at different histidine-rich glycoprotein concentrations were converted to their corresponding apparent heparin concentrations (using the calibration curves) and plotted against the concentrations of free histidine-rich glycoprotein (total histidine-rich glycoprotein minus the amount bound to heparin), sigmoidal curves were obtained (Fig. 1*A*). The shape of the curve obtained for high affinity heparin is compatible with a single association reaction with histidine-rich glycoprotein, but that obtained for the M_r = 4,300 heparin is not. This might indicate that more than one molecule of M_r = 4,300 heparin can interact with different affinities with one molecule of histidine-rich glycoprotein. The dissociation constant of the interaction between histidine-rich glycoprotein and high affinity heparin, determined as the concentration of histidine-rich glycoprotein that decreases the apparent heparin concentration to 50% of its original value, was found to be 7 nM. For histidine-rich glycoprotein and the M_r = 4,300 heparin, a single dissociation constant cannot be determined; a 50% reduction of the apparent heparin concentration was obtained with 36 nM histidine-rich glycoprotein.

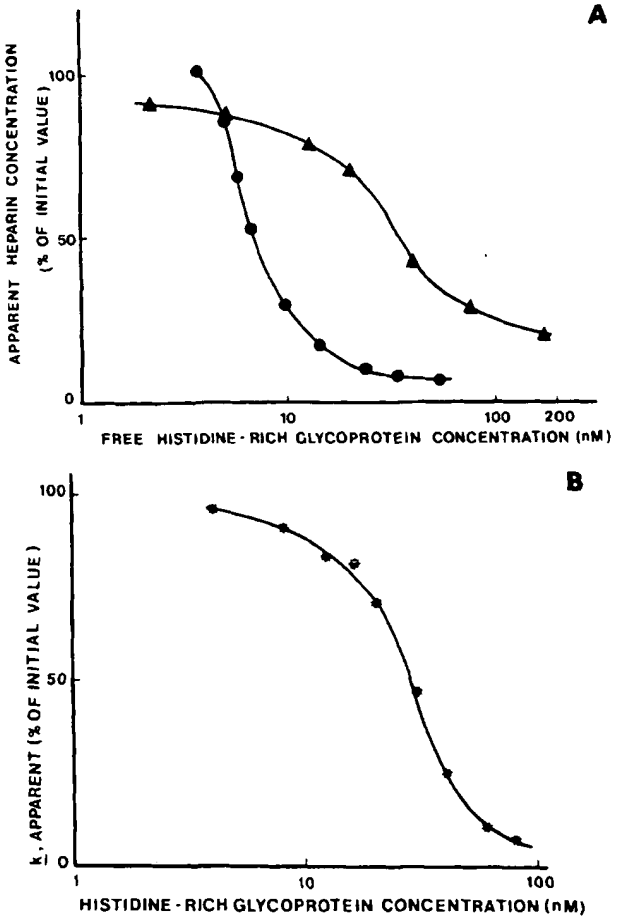

FIG. 1. **Influence of histidine-rich glycoprotein on the inhibition of factor Xa by antithrombin III-heparin.** Histidine-rich glycoprotein (0–200 nM final concentration) was added to a mixture of antithrombin III (107 nM final concentration), S-2222 (0.2 mM final concentration), and different heparin species (high affinity heparin, a M_r = 4,300 heparin fragment, or a covalent heparin-antithrombin III complex) followed by measurements of the factor Xa inhibition. *A*, plot of the apparent heparin concentration (in percentage of the initial concentration) for high affinity heparin (final concentration, 7.3 nM) (●) and for the M_r = 4,300 heparin (final concentration, 32 nM) (▲), *versus* the concentration of histidine-rich glycoprotein (corrected for the amount bound to heparin). *B*, plot of the apparent rate constant (in per cent of the value in the absence of histidine-rich glycoprotein) obtained with the covalent heparin-antithrombin III complex (final concentration 10 nM) (★) *versus* the total concentration of histidine-rich glycoprotein.

The influence of histidine-rich glycoprotein on the apparent rate constant of the inhibition of factor Xa by the covalent heparin-antithrombin III complex is illustrated in Fig. 1*B*. The shape of this sigmoidal curve is also compatible with a single association reaction, and the dissociation constant of the interaction between histidine-rich glycoprotein and the covalent heparin-antithrombin III complex was found to be 29 nM.

Influence of Low Affinity Heparin on the Neutralization of High Affinity Heparin by Histidine-rich Glycoprotein—The apparent rate constant of the inhibition of 1.2 nM factor Xa by a mixture of 107 nM antithrombin III and 7.3 nM high affinity heparin is 36.3×10^{-3} s^{-1}. In the presence of 30 nM histidine-rich glycoprotein, this value is reduced to 2.96×10^{-3} s^{-1}. Addition of low affinity heparin causes a concentration-dependent increase of the apparent rate constant, as illus-

trated in Table I. These findings indicate that low affinity heparin efficiently competes with high affinity heparin for binding to histidine-rich glycoprotein but not to antithrombin III.

Neutralization of Heparin Activity by Histidine-rich Glycoprotein in Human Plasma

Table II shows that the prolongation of the thrombin time following addition of clinical grade or of high affinity heparin was much more pronounced in plasma depleted in histidine-rich glycoprotein (about 5% residual histidine-rich glycoprotein) than in normal plasma. Thus, at a final heparin concentration of 0.250 IU/ml, the plasma depleted in histidine-rich glycoprotein is incoagulable, whereas the thrombin time in normal plasma is not significantly prolonged. When the depleted plasma is reconstituted with purified histidine-rich glycoprotein, the thrombin time shortens dramatically. The results obtained with clinical grade heparin and with high affinity heparin were comparable. These results indicate that histidine-rich glycoprotein efficiently competes with antithrombin III for binding of heparin in plasma, thereby counteracting the anticoagulant activity of heparin. Table III illustrates the effect of low affinity heparin (0–800 nM) on the thrombin times of pooled normal plasma in the presence or in the absence of 40 nM high affinity heparin. Addition of 80 nM low affinity heparin has very little effect on the thrombin time of nonheparinized plasma but markedly prolongs that of plasma containing 40 nM high affinity heparin (from 31 to 112 s). At higher concentrations of low affinity heparin (500–800 nM), the thrombin time reaches a plateau of approximately 145 s, a value which is also obtained with 80 nM high affinity heparin alone. These findings are compatible with the interpretation that low affinity heparin efficiently competes with high affinity heparin for binding to histidine-rich glycoprotein but not to antithrombin III.

Table IV shows that when a fixed amount of heparin is added to plasma depleted in histidine-rich glycoprotein, the factor Xa inhibitory activity decreases with addition of increasing amounts of purified histidine-rich glycoprotein. When EDTA (final concentration, 2.5 mM) is added to the incubation mixture, 95% of the heparin is measured in the plasma reconstituted with histidine-rich glycoprotein to normal plasma levels. The same effect of EDTA is observed using normal plasma. This indicates that the effect of histidine-rich glycoprotein on the anticoagulant activity of heparin is abolished by EDTA.

Mechanism of the Interaction between Histidine-rich Glycoprotein and Heparin

Fig. 2*A* shows that in crossed immunoelectrophoresis the mobility of histidine-rich glycoprotein increases upon complex formation with both low affinity and clinical grade heparin; this effect is completely abolished upon addition of EDTA to the gel. The mobility of the purified histidine-rich glycoprotein in the absence of heparin is the same as in plasma and is not influenced by the presence of EDTA (not shown). Fig. 2*B* shows that the mobility of antithrombin III increases in the presence of clinical grade but not of low affinity heparin, and that this effect is not abolished by EDTA. These findings indicate that the mechanism of complex formation between histidine-rich glycoprotein and heparin is completely different

TABLE I

Influence of low affinity heparin on the interaction between high affinity heparin and histidine-rich glycoprotein

Apparent rate constant (k_{app}) of the inhibition of 1.2 nM factor Xa by a mixture of 107 nM antithrombin III, 30 nM histidine-rich glycoprotein and 7.3 nM high affinity heparin in the presence of different concentrations of low affinity heparin.

Concentration of low affinity heparin	Apparent rate constant, k_{app}
nM	$\times 10^{-3}\ s^{-1}$
0	2.96
3.3	6.00
6.6	21.4
9.9	31.0
0, no HRG	36.3

TABLE III

Combined effect of high affinity heparin (40 nM) and different concentrations of low affinity heparin on the thrombin time (in seconds) of pooled normal plasma

Concentration of low affinity heparin	Thrombin time	
	Without high affinity heparin	In the presence of 40 nM high affinity heparin
nM	*s*	
0	17	31
40	21	79
80	21	112
160	26	102
320	33	121
480	42	146
800	66	140

TABLE IV

Amount of heparin measured in depleted plasma reconstituted with purified histidine-rich glycoprotein by a factor Xa inhibition assay upon addition of clinical grade heparin or a M_r = 4,300 heparin fragment (final concentrations in the incubation mixture, 0.08 and 0.04 IU/ml respectively)

The results are given as a percentage of the heparin measured in histidine-rich glycoprotein-depleted plasma.

Plasma	Clinical grade heparin	M_r = 4,300 heparin
Plasma depleted in histidine-rich glycoprotein	100	100
Depleted plasma reconstituted to 60%	60	82
Depleted plasma reconstituted to 90%	45	72
Depleted plasma reconstituted to 200%	30	55

TABLE II

Influence of the concentration of histidine-rich glycoprotein in human plasma on the thrombin time (in seconds) in the presence of clinical grade heparin or of high affinity heparin

Plasma	Final heparin concentration									
	Clinical grade heparin						High affinity heparin			
	0	0.125	0.250	0.500	0.750	1.0	0.125	0.250	0.500	0.625
	IU/ml plasma						*IU/ml plasma*			
Normal plasma	16.1	16.7	18.1	30.8	61.2	>180	16.9	17.6	24.6	62.4
Plasma depleted in histidine-rich glycoprotein	16.6	44.1	>180	>180	>180	>180	55.9	>180	>180	>180
Depleted plasma reconstituted to 60%	17	21.6	28.2	>180	>180	>180	22.3	37.8	>180	>180
Depleted plasma reconstituted to 90%	17.1	19.4	22.8	99.2	155.2	>180	21.3	24.7	57.6	>180
Depleted plasma reconstituted to 200%	17.2	17.5	19.3	25.6	27.4	95	19.5	19.2	27.2	32.3

from that between antithrombin III and heparin.

The influence of EDTA on the complex formation between histidine-rich glycoprotein and high affinity heparin was further investigated in a kinetic system measuring factor Xa inhibition by antithrombin III. Table V shows that in the presence of increasing concentrations of EDTA the apparent rate constant of the inhibition of factor Xa by a mixture of 107 nM antithrombin III, 30 nM histidine-rich glycoprotein, and 7.3 nM high affinity heparin, increases to reach the value obtained in the absence of histidine-rich glycoprotein (35×10^{-3} s^{-1}). No influence of EDTA on the inhibition of factor Xa in the absence of histidine-rich glycoprotein was observed. Table VI illustrates the effect of addition of divalent metal ions (10 μM final concentration) on the factor Xa inhibition by a mixture of 107 nM antithrombin III, 30 nM histidine-rich glycoprotein (treated with EDTA followed by extensive dialysis), and 7.3 nM high affinity heparin. The apparent rate constant of the factor Xa inhibition decreases upon addition of divalent cations from 18×10^{-3} s^{-1} to 3.0×10^{-3} s^{-1} in the presence of Zn^{2+} or Cu^{2+} and to intermediate values for Fe^{2+}, Ca^{2+}, and Mg^{2+}.

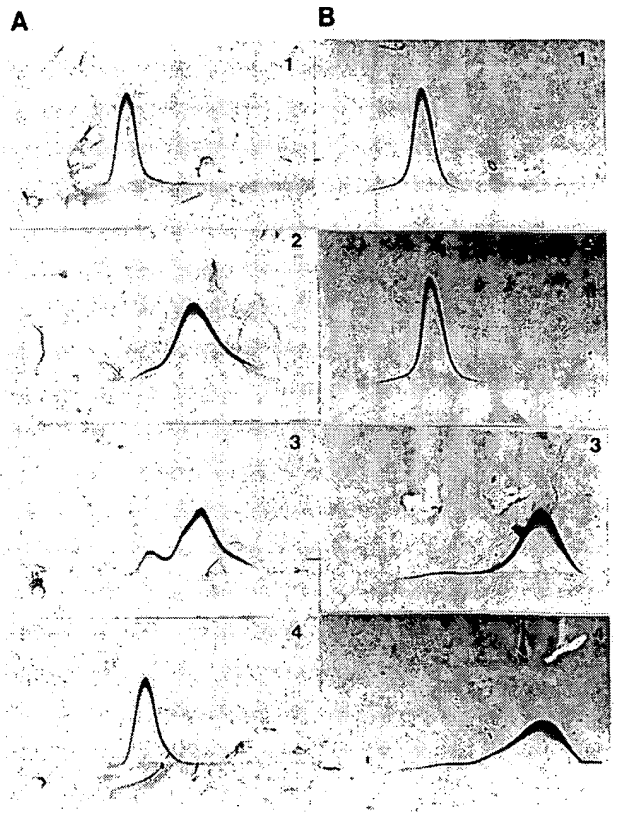

FIG. 2. **Crossed immunoelectrophoresis of purified histidine-rich glycoprotein (*A*) and antithrombin III (*B*) using a monospecific rabbit antiserum.** The antigens were applied in the absence of heparin (*1*), in the presence of 0.2 mg of low affinity heparin/ml of gel (*2*), of 50 IU (0.5 mg) clinical grade heparin/ml of gel (*3*), and in the presence of a mixture of EDTA (50 μM final concentration) and clinical grade heparin (0.5 mg/ml of gel) (*4*).

TABLE V

Influence of EDTA on the interaction between high affinity heparin and histidine-rich glycoprotein

Apparent rate constant (k_{app}) of the inhibition of 1.2 nM factor Xa by a mixture of 107 nM antithrombin III, 30 nM histidine-rich glycoprotein, and 7.3 nM high affinity heparin in the presence of different concentrations of EDTA.

Concentration of EDTA	Apparent rate constant, k_{app}
μM	$\times 10^{-3}$ s^{-1}
0	2.3
5	5.7
7.5	15
10	22
0, no HRG	35
10, no HRG	35

TABLE VI

Influence of divalent metal ions on the interaction between high affinity heparin and histidine-rich glycoprotein

Apparent rate constant (k_{app}) of the inhibition of 1.2 nM factor Xa by a mixture of 107 nM antithrombin III, 7.3 nM high affinity heparin, and 30 nM histidine-rich glycoprotein (treated with EDTA) in the presence of different divalent cations (10 μM final concentration).

Divalent cation	Apparent rate constant, k_{app}
	$\times 10^{-3}$ s^{-1}
None	18
Mg^{2+}	11
Ca^{2+}	8
Fe^{2+}	6
Cu^{2+}, Zn^{2+}	3

DISCUSSION

The present study shows that histidine-rich glycoprotein inhibits the fast neutralization of factor Xa and thrombin by antithrombin III-heparin mixtures. This effect appears to be due to competition between histidine-rich glycoprotein and antithrombin III for binding to heparin. This phenomenon is observed both in purified systems and in plasma. The interaction between histidine-rich glycoprotein and high affinity heparin seems to occur as a single association reaction, while the interaction with a $M_r = 4{,}300$ fragment of heparin is more complex, and occurs with a lower affinity. The covalent complex between heparin ($M_r = 15{,}000$) and antithrombin III also interacts with histidine-rich glycoprotein, suggesting that the binding sites for antithrombin III and histidine-rich glycoprotein in heparin are different.

The interaction between heparin and histidine-rich glycoprotein is abolished by EDTA and restored by different divalent cations. The order of effectiveness is: $Cu^{2+} \simeq Zn^{2+} > Fe^{2+} > Ca^{2+} > Mg^{2+}$, which is the same as that of the affinity of divalent metal ions for binding to histidine-rich glycoprotein (22). These results suggest that the interaction between histidine-rich glycoprotein and heparin occurs via divalent cations bound to the protein, which can be removed by EDTA. It can, however, at present not be excluded that EDTA might interact directly with the heparin binding sites on histidine-rich glycoprotein and that the restoration of the binding of heparin by addition of divalent cations, could be due to removal of EDTA from the protein.

The mechanism of the interaction between heparin and histidine-rich glycoprotein appears to be completely different from that between heparin and antithrombin III. This is substantiated by the finding that EDTA does not influence the binding of heparin to antithrombin III, and that low affinity heparin binds to histidine-rich glycoprotein but not to antithrombin III.

Low affinity heparin was found to compete with high affinity heparin for binding to histidine-rich glycoprotein in purified systems as well as in plasma. This might imply that high affinity heparin could be a more potent antithrombotic agent *in vivo* in the presence of low affinity heparin. Whether the interaction of heparin occurs with the total population of histidine-rich glycoprotein molecules in plasma or only with a fraction saturated with divalent cations, is at present not clear.

The finding that the inhibition of factor Xa by antithrombin

III in the presence of a M_r = 4,300 fragment of heparin is less disturbed in the presence of histidine-rich glycoprotein might be important for the future use of low molecular weight heparin fragments.

Interaction of heparin with several proteins, including fibrinogen (7), albumin (7), fibronectin (8), lipoprotein lipase (9), α_1-acid glycoprotein (10), and platelet factor 4 (11, 12) has been observed, but it remains unclear whether any of these interactions are of physiopathological importance. The present study shows that histidine-rich glycoprotein is an efficient competitor of antithrombin III for the binding of heparin, resulting in neutralization of its anticoagulant activity in plasma. The potent effect of histidine-rich glycoprotein suggests that it might play a role in the modulation of the anticoagulant properties of heparin *in vivo*.

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